MOLECULAR REPRODUCTION AND DEVELOPMENT 35:244–250 (1993)

Binding Characteristics and Immunolocalization of Porcine Seminal Protein, PSP-I

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ABSTRACT PSP-I, a 13 kDa protein purified from boar seminal plasma, was found to have about 50% amino acid sequence homology with a family of zona pellucida-binding proteins known as spermadhesins. These proteins are produced by the accessory gland(s) of the male reproductive tract and coat the spermatozoa during ejaculation. In this study, we have investigated the possible biological functions of PSP-I using a solid-phase protein binding assay and its site of synthesis using both Western blot and immunocytochemical analyses. PSP-I was found to bind a number of proteins including endo-β-galactosidase digested ZP3, soybean trypsin inhibitor, IgA, IgG and α-casein, indicating that it may have multiple functions. The protein or carbohydrate structures were not critical in the binding, since polyvinyl sulfate could effectively inhibit the binding of PSP-I to these proteins. Western blot analysis using specific antiserum to PSP-I showed that the protein was present in the seminal vesicle but not in the testes, epididymis or prostate. The protein was revealed by immunocytochemical analysis in the epithelium of seminal vesicles but not in the testes or the epididymis. It is concluded that PSP-I is synthesized by the epithelium of the seminal vesicles, secreted into the semen during ejaculation, and may be involved in various reproductive functions, such as preventing premature acrosome reaction and immunosuppression.

Key Words: Spermadhesin, Seminal vesicle, Semen, Immunocytochemistry

INTRODUCTION

Two major 13-14 kDa proteins, designated PSP-I and PSP-II, have been recently purified from porcine seminal plasma in our laboratory. The complete amino acid sequence of PSP-I and a stretch of 13 residues of N-terminal sequence of PSP-II have been determined [Rutherfurd et al., 1992]. These two proteins form both homodimers and heterodimers. Both exist in multiple forms, differing in amino acid sequence and/or in the carbohydrate moiety. Independently, two porcine seminal proteins having identical N-terminal sequences with PSP-I and PSP-II have also been reported by Parry et al. [1992]. On the other hand, a group of nine 15-18 kDa zona pellucida binding proteins have been purified from the plasma membranes of ejaculated boar spermatozoa by four groups of investigators [Jonakova et al., 1991; Sanz et al., 1992a; Hanqing et al., 1992; Moos et al., 1992; Parry et al., 1992]. These proteins are distinct from PSP-I and PSP-II, and most of them have N-terminal sequence of Ala-Gln-Asn. Sanz and coworkers [1992b] named this protein family "spermadhesin", since they are produced by the accessory gland(s) of the male reproductive tract, mixed with sperm during ejaculation, and coated on the sperm surface. The amino acid sequences of three of the spermadhesins (designated AQN-1, AQN-3 and AWN) have been reported [Sanz et al., 1991, 1992b, 1992c]. The amino acid sequence of AWN is 53% and 48% similar to that of AQN-1 and AQN-3 respectively, whereas AQN-1 and AQN-3 share 59% sequence similarity. On the other hand, PSP-I shows 50%, 53% and 47% amino acid similarities to AWN, AQN-1 and AQN-3 respectively. Thus, PSP-I (and probably PSP-II) may belong to the same spermadhesin family.

These proteins are found to bind zona pellucida, and thus may be involved in sperm-egg recognition [Jonakova et al., 1991; Sanz et al., 1992a; Hanqing et al., 1992; Moos et al., 1992; Parry et al., 1992]. Some of them are also found to bind acrosin inhibitor and may have a function in preventing premature capacitation and acrosome reaction [Sanz et al., 1992d]. On the other hand, β-microseminoprotein, a 94-residue seminal protein produced by human prostate, is shown to bind immunoglobulin, indicating that it may function as an immunosuppressive factor [Liang et al., 1991]. Furthermore, a group of seminal proteins from bovine seminal vesicles are shown to bind gelatin and apolipoprotein A-I, although their physiological functions remain to be determined [Manjunath et al., 1987; 1989]. We have investigated these possible biological functions of PSP-I using a solid-phase protein binding assay and report herein some binding characteristics of PSP-I. In addition, we also report the result of immunolocalization of PSP-I.

Received November 27, 1992; accepted January 18, 1993.
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MATERIALS AND METHODS

Materials

Porcine seminal protein, PSP-I, was purified from boar seminal plasma as previously described [Rutherfurd et al., 1992]. Native zona pellucida protein, ZP3, and endo-β-galactosidase-digested ZP3 were prepared as described earlier [Yurewicz et al., 1987]. Antiserum against highly purified PSP-I was raised in adult New Zealand White rabbit without coupling to carrier protein. The immunization and bleeding protocols were similar to that previously described for the generation of antipeptide antisera [Deb et al., 1989].

Preparation of Biotinylated PSP-I

The procedure of Bullesbach and Schwabe [1990] was used. Briefly, 7 mg of PSP-I was dissolved in 1 ml of 0.2 M NaH_2PO_4/NaOH, pH 6.0, and was mixed with 6 μl of biotinamidocaproate N-hydroxysuccimide ester (Sigma, 50 mg/ml in dimethylformamide) for 2 h at 25°C. The mixture was then adjusted to 1 M acetic acid with glacial acetic acid and desalted on a 1.5 × 30 cm column of Sephadex G25 equilibrated with 1 M acetic acid. The biotinylated protein peak was pooled, diluted with deionized water and lyophilized twice. The protein was redissolved in Tris buffered saline (TBS) containing 0.02% sodium azide at a concentration of 1 mg/ml and stored at 4°C.

Solid Phase Protein Binding Assay

An ELISA procedure was used for the binding studies. Microtiter plates were coated with various amounts (3.91-500 ng/well) of proteins in 100 μl of 50 mM sodium carbonate buffer (pH 9.6) at 4°C for 7 h, and then blocked with 200 μl/well of 1% BSA in TBS at 4°C for 16 h. After washing the plate 4 times with washing buffer (0.05% Tween 20 in TBS), 100 μl of 1 μg/ml biotinylated PSP-I (diluted with 0.1% BSA in TBS) was then added to each well, and the plate was allowed to incubate at 25°C for 3 h. Microtiter plates were then washed, and incubated with 100 μl/well of 1 μg/ml streptavidin-alkaline phosphatase conjugate (Pierce, diluted with 0.1% BSA in TBS) at 25°C for 1 h. The plate was washed again and incubated with 100 μl/well of substrate solution (Sigma 104) at 37°C. The color developed was measured at 410 nm with a micro-ELISA Autoreader (Dynatech Laboratories Inc.). Absorbance due to nonspecific binding was subtracted from each measurement by the average absorbance of wells coated with 500 ng BSA.

Preparation of Tissue Extracts and Electrophoresis

Pig tissues were extracted by homogenization with 3 volumes (w/v) of extraction buffer [50 mM Tris HCl, pH 8.5 (4°C), 5 mM EDTA, 100 μg/ml PMSF 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin] using a Polytron at medium speed for 1 min. The homogenates were then centrifuged twice at 4°C for 20 min at 20,000 × g. The supernatants were stored at −20°C until use. Protein concentration in the extracts was determined with the BCA Protein Assay Reagent (Pierce). For electrophoresis, a 15% SDS-polyacrylamide gel was used [Laemmli, 1970]. About 23 μg protein from each extract was boiled with 30 μl of sample buffer for 5 min before electrophoresis.

Western Blot Analysis

The procedure of Burnette [1981] was used with minor modifications. The separated proteins were electrophoretically transferred to nitrocellulose, blocked with 5% nonfat dry milk, and reacted with rabbit antiserum against PSP-I. After thorough washing, the blot was incubated with a mixture of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) and streptavidin-alkaline phosphatase conjugate (Pierce). The PSP-I and biotinylated molecular weight markers were then visualized with chromogenic substrates (nitro blue tetrazonium and 5-bromo-4-chloro-3-indolyl phosphate).

Immunolocalization of PSP-I

The tissues were fixed in Bouin's fixative, dehydrated, cleared, embedded in paraffin, and sectioned at 7 μm. The tissue sections were stained for the presence of PSP-I using an avidin-biotin immunoperoxidase kit for rabbit IgG (Zymed Laboratories, South San Francisco, CA) as previously described [Campbell et al., 1989]. The porcine PSP-I antiserum was used at a final dilution of 1:200. Control sections were examined using preimmune serum.

RESULTS

Biotinylated PSP-I was found to bind a number of immobilized proteins including endo-β-galactosidase digested ZP3, soybean trypsin inhibitor, IgA, IgG and α-casein (Fig. 1). Among them, α-casein had the highest affinity. The binding was highly reproducible, and biotinylation did not interfere with the binding. In another experiment, biotinylated human, mouse and pig IgG were found to bind equally well to immobilized native PSP-I (data not shown). However, biotinylated PSP-I did not bind to native ZP3 and IgM (Fig. 1), nor to gelatin and apolipoprotein AI (data not shown).

The fact that PSP-I can bind to a number of structurally distinct proteins indicates that the amino acid sequence may not be important for the binding. The involvement of a lectin-like binding was thus investigated by competing the binding of biotinylated PSP-I to immobilized α-casein with various carbohydrates. Simple sugars such as glucose, fucose, galactose, mannose, lactose, melibiose and N-acetylglucosamine had little effect on the binding of biotinylated PSP-I to α-casein (data not shown). Some complex carbohydrates such as heparin, dextran and dextran sulfate also failed to compete with PSP-I (Fig. 2). Fucoidan and chondrotin sulfate B, at 1 mM concentrations, reduced the binding of PSP-I to α-casein to 76.4% and 44.4%, respectively. The most effective competitor was polyvinyl sulfate, which reduced the binding to 9.9% at 0.1 mM. At this concentra-

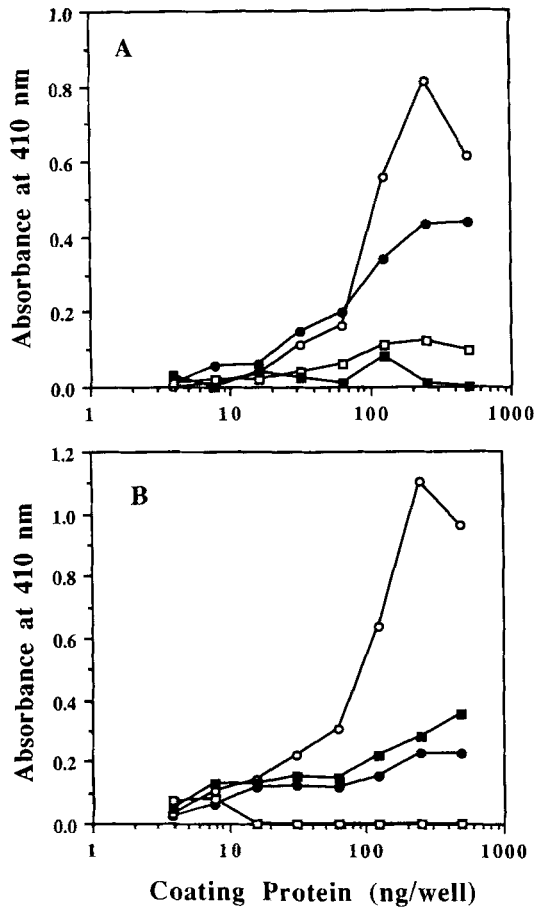

Fig. 1. Binding of biotinylated PSP-I to various immobilized proteins. (A) Microtiter wells were coated with 3.91-500 ng of bovine α-casein (open circle), pig IgG (filled circle), human IgA (open square) and bovine IgM (filled square), and incubated with 1 μg/ml of biotinylated PSP-I. Bound PSP-I was detected by an ELISA procedure as described in Materials and Methods and expressed as relative absorbance at 410 nm. The protein concentrations were those of the coating solutions. Absorbance due to nonspecific binding was subtracted from each point by the average absorbance of wells coated with 500 ng BSA. Each point represents the mean of duplicates. (B) Microtiter wells were coated with α-casein (open circle), soybean trypsin inhibitor (filled circle), native ZP3 (open square), and endo-β-galactosidase-digested ZP3 (filled square). Experimental conditions are the same as in (A).

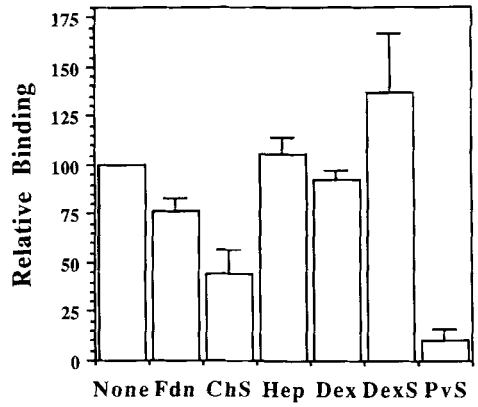

Fig. 2. Inhibition of α-casein-PSP-I binding by various carbohydrates and sulfated compounds. Microtiter wells were coated with 500 ng α-casein and incubated with 1 μg/ml of biotinylated PSP-I in the presence of various carbohydrates and sulfated compounds. Bound PSP-I was detected by an ELISA procedure as described in Materials and Methods. The results were expressed as relative binding with the control set at 100%. Each relative binding is the mean of four experiments ± the standard deviation for each inhibitor. Fdn, 1 mM fucoidan; Chs, 1 mM chondroitin sulfate B; Hep, 1 mM heparin; Dex, 1 mM dextran (M_r = 8,800); DexS, 0.1 mM dextran sulfate (M_r = 500,000); and PvS, 0.1 mM polyvinyl sulfate.

tion, polyvinyl sulfate also effectively inhibited the binding of PSP-I to IgG, IgA, soybean trypsin inhibitor and endo-β-galactosidase digested ZP3 (data not shown).

Western blot analysis of extracts of porcine semen, prostate, seminal vesicle, testes and epididymis using specific antiserum to PSP-I showed that only the seminal vesicle and semen contained immunoreactive PSP-I (Fig. 3, bottom panel, lanes 1 and 3), indicating the seminal vesicles as a source of PSP-I. All five protein bands in the cluster were detected by the antiserum, although the intensities were not proportional to those of the stained gel. It is known that some of these protein bands contain more than one protein of the same size and that PSP-I has multiple forms due to the heterogeneity of the carbohydrate moiety [Rutherfurd et al., 1992]. Immunocytochemical analysis of sections of porcine seminal vesicle, testes and epididymis using antiserum using PSP-I showed that only the epithelium of the seminal vesicles was stained (Fig. 4, micrograph b), while those of the testes and epididymis were negative (Fig. 4, micrographs c and d). Immunostaining using preimmune serum on sections of seminal vesicle also gave negative result (Fig. 4, micrograph a). This indicated that the immunostaining of seminal vesicle by PSP-I antiserum was specific.

DISCUSSION

In this report we have demonstrated the binding of PSP-I to a number of proteins such as α-casein, IgG, IgA, endo-β-galactosidase-digested ZP3 and soybean trypsin inhibitor. This suggests that PSP-I may have multiple functions. It is intriguing to note that PSP-I binds to endo-β-galactosidase digested ZP3, but not to native ZP3. However, not all spermadhesins bind to zona pellucida. Three members of the spermadhesin family, namely AQN-1, AQN-3, and AWN, bind to zona pellucida, but AQN-2 does not [Sanz et al., 1992d]. It seems that, like AQN-2, PSP-I by itself may not be involved in the initial stage of sperm-egg recognition. Nevertheless, the involvement of PSP-I in the sperm-egg recognition cannot be ruled out, as it has been suggested that many different ligands found on spermatozoa may function at different stages of fertilization [Jones, 1990]. Interaction among members of the spermadhesin family may also play an important role. How-

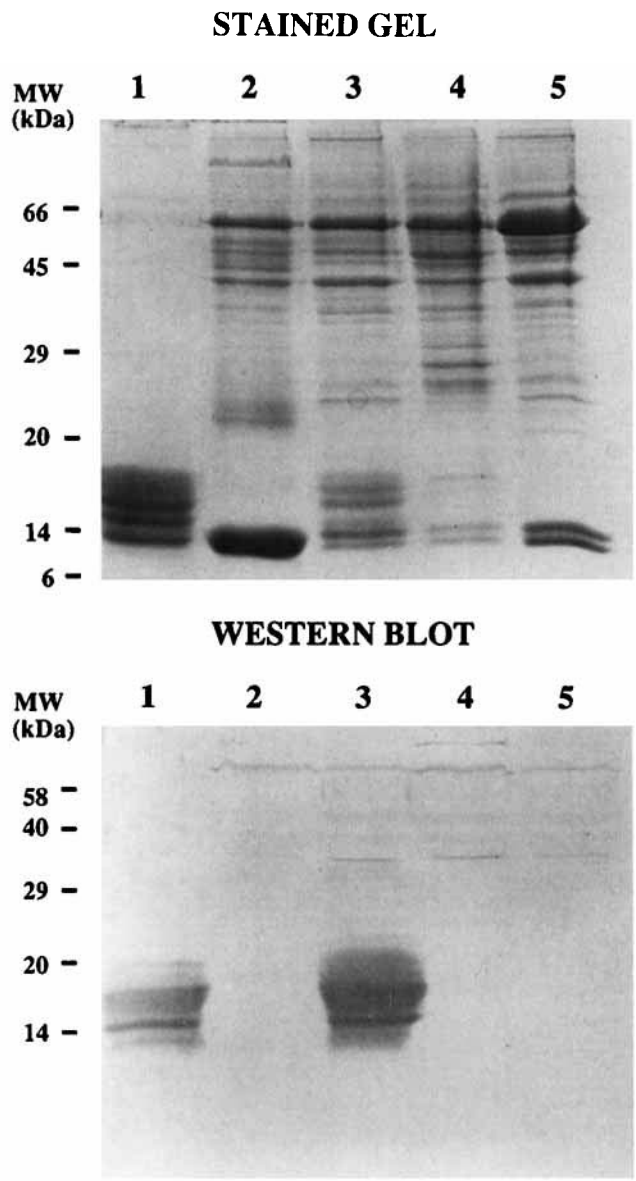

Fig. 3. Western blot analysis of PSP-I in extracts of pig tissues. Approximately 23 μg protein from extracts of pig semen (**lane 1**), prostate (**lane 2**), seminal vesicle (**lane 3**), testis (**lane 4**) and epididymis (**lane 5**) were boiled in 30 μl of sample buffer containing 2-mercaptoethanol for 5 min. and analyzed on a 15% SDS-polyacrylamide gel. (Top) Coomassie stained gel. (Bottom) Western blot analysis of proteins transferred from an identical gel using specific antiserum against PSP-I.

ever, since the amino acid sequence of AQN-2 is not known, it is difficult to draw any conclusion for the binding of PSP-I to ZP3 on the basis of sequence homology with the other members of the spermadhesin family. On the other hand, AQN-1 and AWN, but not AQN-3, have been shown to bind acrosin inhibitor and soybean trypsin inhibitor [Sanz et al., 1992d]. PSP-I can also bind to soybean trypsin inhibitor, indicating that it may also bind to acrosin inhibitor and play a role in preventing premature acrosome reaction, and that it is functionally more similar to AQN-1 and AWN than to AQN-3. This is consistent with the amino acid sequence homology among these spermadhesins. PSP-I shows 50%, 53% and 47% amino acid sequence similarities to AWN, AQN-1 and AQN-3, respectively.

It is interesting to note that PSP-I binds to immunoglobulins. It binds to IgG of various species and human IgA, but not bovine IgM. PSP-I may function as an immunosuppressive factor protecting the spermatozoa from immunological attack during their transport in the female reproductive tract. The presence of immunosuppressive factors in boar semen have been reported [Stanek et al., 1985; Bouvet et al., 1987]. However, they have not been well characterized. One was shown to be a secretory protein from seminal vesicle, but its molecular weight was not reported [Stanek et al., 1985]. The molecular weight of the other was estimated to be 100–110,000 by gel filtration [Bouvet et al., 1987]. This may be too big to be PSP-I, however, the possibility that this may represent an aggregated form of PSP-I has not been ruled out. An immunoglobulin-binding protein was also reported in human seminal plasma [Kamada et al., 1991]. This immunoglobulin-binding factor was found to be identical to human β-microseminoprotein, a protein produced by the prostate and secreted into the semen [Liang et al., 1991]. The binding of biotinylated pig or human IgG to immobilized human β-microseminoprotein has been confirmed in our laboratory (data not shown). However, since PSP-I shares no sequence homology with β-microseminoprotein [Rutherfurd et al., 1992; Akiyama et al., 1985], it is difficult to speculate on the mechanism of immunoglobulin binding.

Bovine seminal proteins, PDC-109 and BSP-A3, are able to bind gelatin [Manjunath et al., 1987] and apolipoprotein A-I [Manjunath et al., 1989], although the physiological significance remains to be determined. Unlike these bovine seminal proteins, PSP-I failed to bind gelatin and apolipoprotein A-I. PSP-I shares little sequence homology with the bovine seminal proteins, PDC-109 and BSP-A3 [Rutherfurd, et al., 1992; Esch et al., 1983; Seidah et al., 1987]. These may represent evolutionarily divergent molecules, serving special reproductive functions in different species.

The binding of PSP-I to α-casein is intriguing, as there are no obvious physiological functions. α-casein was initially tested as a blocking agent, but was found to have the highest affinity for PSP-I. The binding of PSP-I to α-casein can be inhibited by fucoidan, chondrotin sulfate B and polyvinyl sulfate. Fucoidan, a sulfated fucose-containing polysaccharide, has been shown to inhibit the binding of spermadhesins to zona pellucida glycoproteins [Jonakova et al., 1991; Sanz et al., 1992a; Parry et al., 1992]. It is postulated that spermadhesins mediate the binding of sperm to the zona pellucida through a lectin-like binding mechanism. However, our results show that the carbohydrate structure is not required, since synthetic sulfated polymer such as polyvinyl sulfate strongly inhibits the binding

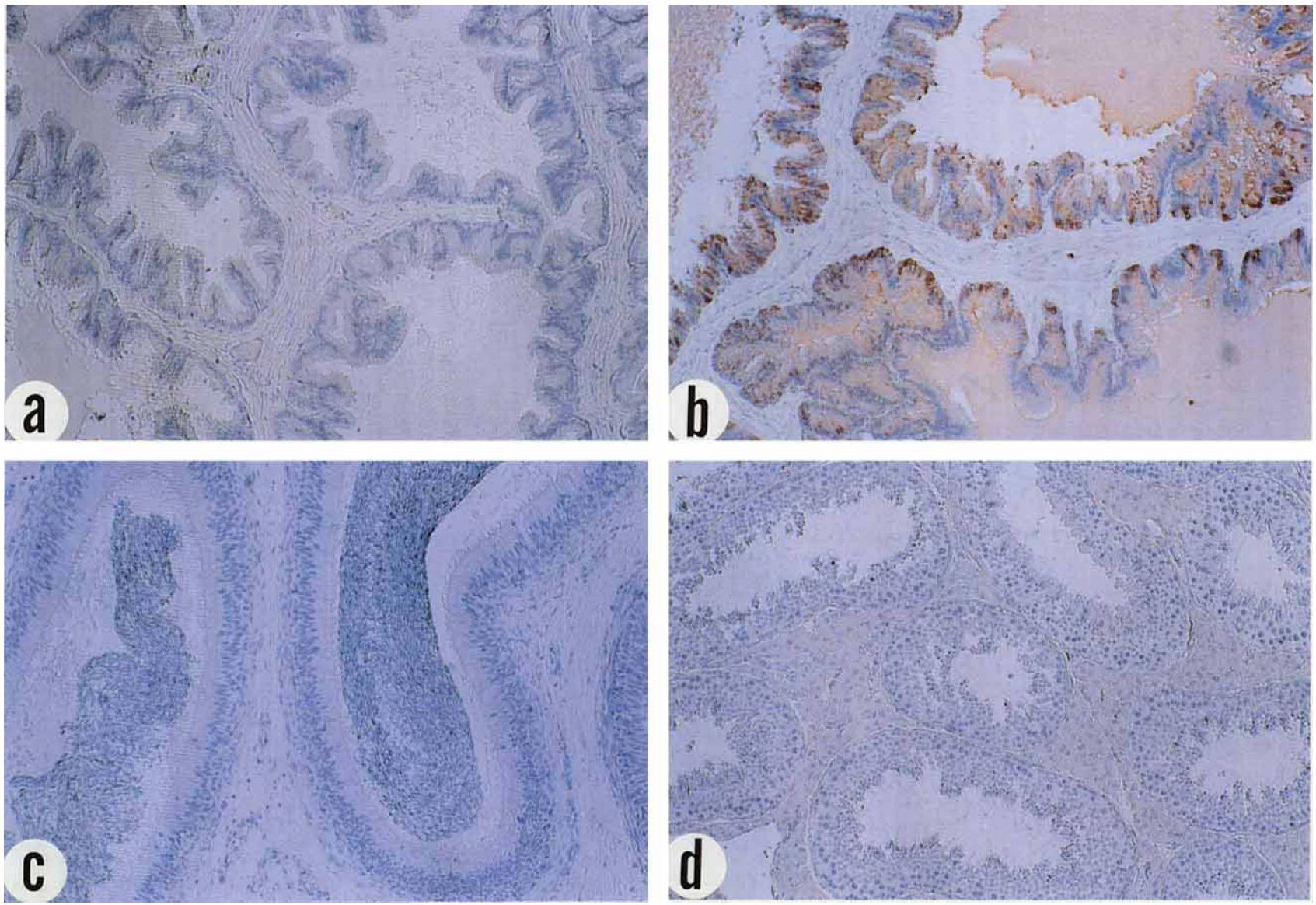

Fig. 4. Immunocytochemical analysis of PSP-I within sections of pig tissues. The sections were stained for the presence of PSP-I using an avidin-biotin immunoperoxidase kit for rabbit IgG. PSP-I was detected with a rabbit antiserum against PSP-I. Micrographs **a** and **b,** seminal vesicles; micrograph **c,** epididymis; micrograph **d,** testis. Micrographs b, c and d were incubated with antiserum to PSP-I, while micrograph a was incubated with preimmune serum. The brown color in the epithelium of seminal vesicles (micrograph b) indicates a positive immunoreactive product. Magnification is 100× for all micrographs.

of PSP-I to α-casein. On the other hand, sugars which are commonly used as eluants in lectin chromatography and other sulfated carbohydrates such as heparin and dextran sulfate are not effective. It seems that the only requirement for the binding of PSP-I to α-casein and other proteins is the presence of sulfate groups in the correct spatial orientation. Therefore, PSP-I may be referred as a polysulfate binding protein rather than a lectin. Similar conclusions have been drawn for bindin, an adhesive protein from sea urchin sperm [DeAngelis and Glabe, 1987] and boar proacrosin [Jones, 1990]. The sulfate groups seem to be important, since polyvinyl sulfate but not polyvinyl phosphate can inhibit the binding of low Mr porcine seminal plasma proteins to zona pellucida glycoproteins [Parry et al., 1992].

The results of the Western blot and immunocytochemical analyses indicate that PSP-I is synthesized by the epithelium of the seminal vesicles and secreted into the semen during ejaculation. The results are in accordance with the report that spermadhesin AWN is present in seminal vesicle fluid and on the surface of ejaculated spermatozoa, but not in epididymal extracts [Sanz et al., 1992a]. The immunolocalization of ACR.3 antigen in seminal vesicle epithelium has also been reported [Moos et al., 1992]. However, the ACR.3 antigen is not PSP-I; it is likely to be a member of the spermadhesin AQN subfamily, since it's N-terminal sequence begins with Ala-Gln-Asn [Moos et al., 1990; Sanz et al., 1991, 1992b]. In other species, however, sperm adhesive proteins of similar size and binding characteristics may be produced by different organs. For example, the rabbit sperm membrane autoantigen, RSA, is produced by testes [O'Rand et al., 1988]. The human β-microseminoprotein, on the other hand, is produced by the prostate [Ito et al., 1989].

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Fuller Bazer for providing the boar semen used in the purification of PSP-I, and Deborah Kwok for the advice in the solid-phase protein assay. This work was supported in part by grants from the Biomedical Research Support Grant Program of NIH (BRSG S07 RR05373) and American Cancer Society (#IN-115).

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